Sex Plant Reprod (1991) 4:104–109

Sexual Plant Reproduction

Responses of tobacco pollen to high humidity and heat stress: viability and germinability in vitro and in vivo

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Summary. Responses of pollen grains of *Nicotiana tabacum* to high humidity (95% RH, 4 h) and temperature (38°/45° C, 4 h) stresses were investigated. Pollen grains were subjected to only RH or only temperature, or to both of these stresses. Their viability was assessed on the basis of the fluorochromatic reaction (FCR) test, and vigour was assessed on the basis of the time taken for in vitro germination as well as on the emergence of pollen tubes through the cut end of semi-vivo implanted styles. None of the stress conditions affected pollen viability and high RH or high temperature stress did not individually affect pollen vigour. However, pollen vigour was markedly affected when both the stresses were given together. Pollen grains subjected to high RH at 38° C took a longer time to germinate in vitro and the pollen tubes emerged later from the cut end of the semi-vivo styles; division of the generative cell was also delayed. Pollen grains subjected to high RH at 45° C failed to germinate in vitro, but did germinate on the stigma. Many pollen tubes subjected to this treatment showed abnormalities, and the growth of pollen tubes in the pistil was much slower than that observed in other treatments. Pollen samples subjected to all of the stress conditions were able to induce fruit and seed set. The implications of these results on the relationship between the FCR test and viability, and between viability and vigour, especially in stressed pollen, are discussed.

Key words: High humidity and temperature stress – *Nicotiana tabacum* – Tobacco – Pollen viability – Vigour – Semi-vivo technique

Introduction

Environmental stresses prevailing during pollen development, germination and pollen-tube growth affect the functioning of the pollen and eventual fruit and seed set. In recent years there have been many studies on the effect of different stresses on pollen germination and/or pollen-tube growth. The responses of pollen to desication stress have been studied in a range of systems (Shivanna and Heslop-Harrison 1981; Hoekstra 1986; Crowe et al. 1989), and the effects of high temperature on pollen responses have been studied (Xiao and Mascarenhas 1985; Frova et al. 1987; Herpen et al. 1988; Frankis and Grayson 1990) in connection with the synthesis of heat shock proteins. Prolonged high humidity stress given to mature anthers prevents their dehiscence and markedly reduces the viability of the pollen (Linskens and Cresti 1988, 1989).

Pollen grains are frequently subjected to humidity and temperature stress after being shed but before landing on the stigma. This aspect has so far not received serious consideration. In an earlier paper (Shivanna and Cresti 1989) we have shown that prolonged high humidity (95% RH for up to 5 h) at 38° C does not affect pollen viability as assessed by fluorochromatic reaction (FCR) test, but does significantly reduce pollen vigour in terms of the time taken for in vitro germination. In this paper we report the results of addition experiments on the effects of high RH and temperature (38° C and 45° C) on pollen viability and in vitro germinability. Studies have also been extended to pollen-tube growth in vivo by using the semi-vivo technique and fruit and seed set.

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Material and methods

Plants of *Nicotiana tabacum* L. growing in a greenhouse of the Botanical Garden of the University of Siena were used as the pollen source. Pollen grains were collected from freshly dehisced anthers and used the same day. The moisture content of the pollen on different days ranged from 16%–19%. The pollen grains were subjected to humidity and heat stress as described earlier (Shivanna and Cresti 1989): They were uniformly spread on slides and maintained under high RH (95%) in petri plates lined with moist filter paper, in the dark, at the ambient laboratory temperature (20° ± 2° C), at 38° ± 1° C or at 45° ± 1° C. Pollen samples were removed after 4 h and tested for viability by means of the fluorochromatic reaction (FCR) test Heslop-Harrison and Heslop-Harrison 1970),

and for in vitro germination (Shivanna and Cresti 1989). The germination medium contained sucrose (10%), boric acid (1 m*M*) and calcium nitrate (1 m*M*). One set of pollen grains was subjected to dry heat (by keeping the slide spread with pollen grains at 38° or 45° C) or only high RH (at 20° ± 2° C) in the dark for 4 h. Each experiment was repeated at least three times. From each replicate 200-300 pollen grains were scored for viability and in vitro germination.

The stressed pollen samples were also used for studying pollen germination and pollen-tube growth in the pistil by means of the semi-vivo technique (see Shivanna et al. 1988; Kandasamy and Kristen 1987). Flower buds just before anthesis and anther dehiscence were brought to the laboratory, emasculated and maintained with their cut end dipped in water until anthesis. The pistils were pollinated by repeatedly bringing the stigma in contact with the pollen spread on a slide. After a 3 h pollination period, the stigma together with 2 or 10 mm of the style was cut with a fresh blade and implanted in an agar medium containing 0.8% agar and other constituents of the germination medium set in a petri plate. The implanted pistils were maintained under high RH in the dark at the ambient laboratory temperature and observed for the emergence of pollen tubes from the cut end of the style. After emergence of the pollen tubes, the pistils were fixed in acetic alcohol (glacial acetic acid:ethanol, 1:3), and the number of emerged pollen tubes from the cut end of the styles were counted. The pollen tubes were the stained with DAPI (Coleman et al. 1981; Hough et al. 1985) and observed under the fluorescence microscope for the division of the generative cell. Some of the semi-vivo pollinated pistils were fixed in acetic alcohol, cleared in 8 N NaOH, and used to study pollen germination and pollen-tube growth through aniline blue fluorescence (Linskens and Esser 1957; Dumas and Knox 1983).

Pollen grains treated with different stress conditions were also used to carry out pollinations of emasculated and bagged flowers maintained on plants. The fruit and seed set was studied after 4 weeks, when the fruits started turning brown.

Results

Figure 1 presents the results of the effects of high RH and temperature stress on FCR and in vitro germination. None of the treatments significantly affected the

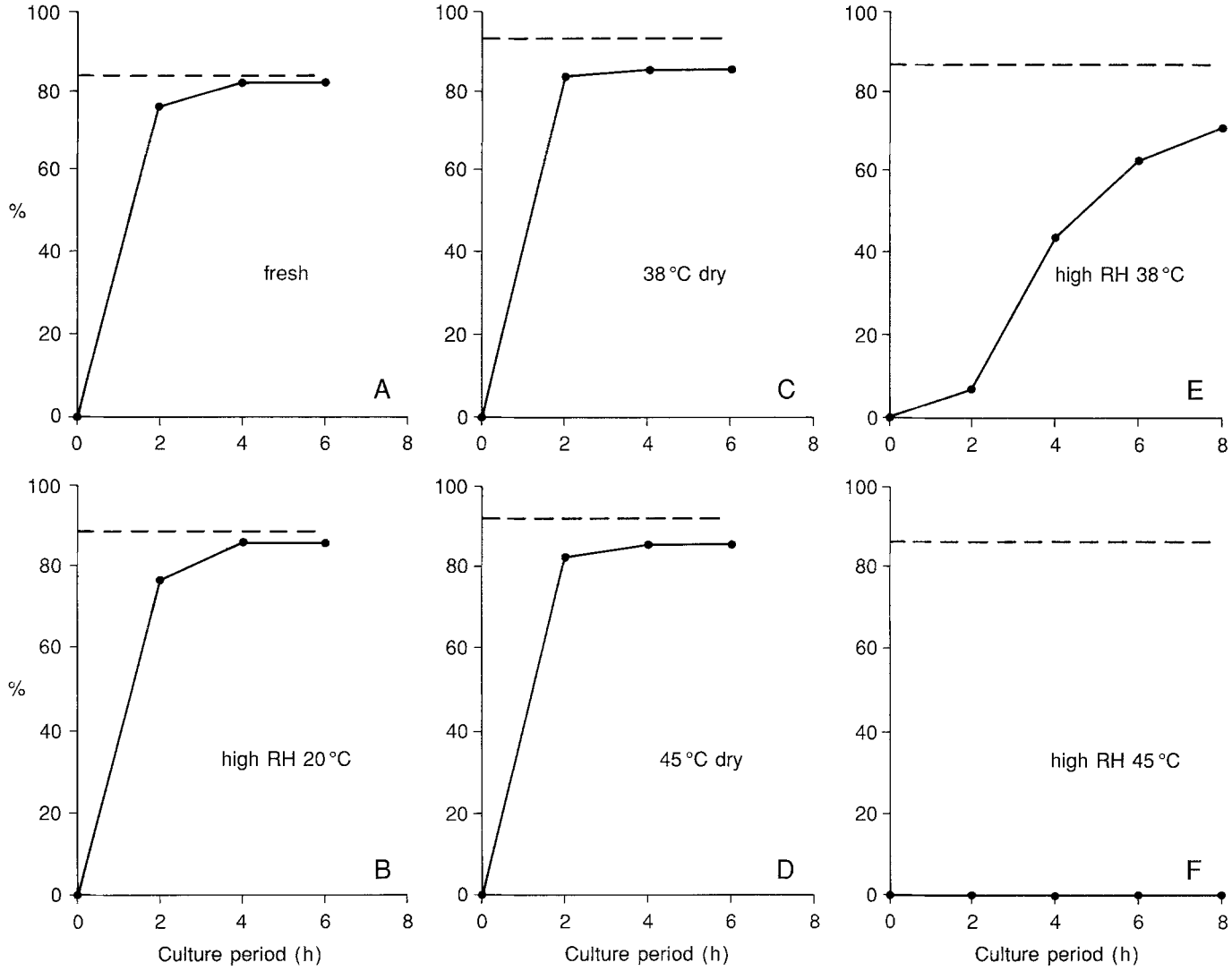

Fig. 1 A–F. FCR and in vitro germination responses of pollen samples subjected to high RH and heat stresses. In all of the treatments FCR was not significantly affected and remained above 80%. In **A–D**, maximum in vitro germination was reached in 1–2 h; in **E** it reached 70% in 8 h and in **F** pollen grains did not germinate. * Pollen grains exposed to high RH for 1 h before being tested for FCR and in vitro germination

FCR score, and neither high RH at the laboratory temperature nor high temperature (38/45° C) on dry pollen affected, in vitro germination. However, high RH combined with high temperature (38/45° C) did significantly affect in vitro germination. Unlike other treatments in which pollen germination reached its maximum (comparable to FCR value) within 2–4 h, in the high RH at 38° C treatment there was hardly any germination within 2 h, and even by 8 h it had only reached about 70%. Pollen samples treated with high RH at 45° C failed to germinate in vitro even after 12 h. Under the cultural conditions used, the generative cell failed to divide in vitro even after 15 h

Table 1 presents the number of pollen tubes that emerged from 2-mm- and 10-mm-long semi-vivo styles subjected to different treatments. As the FCR and in vitro germination responses of pollen grains subjected to 38° C and 45° C in the dry state were very similar to those of fresh pollen, these two pollen samples were not used in semi-vivo experiments. In the 2-mm-long styles pollinated with fresh pollen as well as pollen subjected to high RH at the laboratory temperature, more than 100 pollen tubes emerged within 10 h after pollination (Fig. 3A). In styles pollinated with pollen subjected to high RH at 38° C only about 7 tubes/pistil emerged; the number increased to about 50 by 12 h and the number of tubes comparable to those pollinated with fresh pollen emerged only at 14 h (Fig. 3B–D). In pistils pollinated with pollen subjected to high RH at 45° C, no pollen tubes emerged even by 14 h.

One set of semi-vivo-2-mm-long styles were fixed 12 h after pollination; pollen-tube bundles were stained with DAPI and used to score the percentage of pollen tubes containing sperm cells/generative cell. Over 65% of the pollen tubes of styles pollinated with pollen subjected to high RH at the laboratory temperature showed the sperm cells; the generative cell was in a divisional stage in most of the remaining tubes (Fig. 2). On the other hand, only 22% of the pollen tubes of styles pollinated with pollen subjected to high RH at 38° C showed sperm cells; the generative cell was in division or had not yet entered division in the remaining tubes.

Table 1. Number of pollen tubes emerging from the cut end of 2- and 10-mm-long styles pollinated with pollen subjected to different stress conditions. Average of 12 pistils for each treatment

Treatment	Mean number of pollen tubes/pistil			
	2-mm style		10-mm style	
	10 h	14 h	16 h	24 h
Fresh pollen[a]	>100[b]	>100	>100	>100
High RH at laboratory temperature	>100	>100	>100	>100
High RH at 38° C	6.8	>100	10.4	>100
High RH at 45° C	0	0	0	0

[a] Pollen grains exposed to high RH for 1 h before pollination
[b] Pollen tube number could not be counted accurately beyond 100

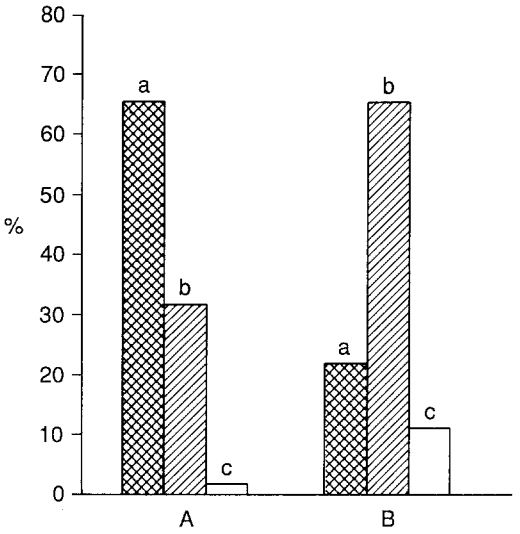

Fig. 2. Percentage of pollen tubes that emerged from the cut end of a 2-mm-long style within 12 h, showing sperm cells (*a*), generative cell in division (*b*) and generative cell not yet dividing (*c*). Pollinations with pollen treated with high RH at laboratory temperature (*A*) and with high RH at 38° C (*B*). Average of 400 tubes for each treatment

In the 10-mm-long styles more than 100 tubes also emerged and grew 1–1.5 mm in the agar medium within 16 h in pistils pollinated with fresh pollen and pollen subjected to high RH at the laboratory temperature (Table 1).In treatments involving high RH at 38° C, it took 24 h to attain a number of pollen tubes comparable to what emerged from the control. Almost all of the pollen tubes which emerged under the different treatments with 10-mm styles showed sperm cells.

In the treatment involving high RH at 45° C, no pollen tubes emerged even by 24 h. These pistils were fixed and studied for pollen germination and pollen-tube growth. Interestingly, all of the pistils showed good pollen germination on the stigma (Fig. 3E). However, some pollen tubes had failed to enter the stigma and showed abnormalities such as thick callose deposition in the tube, curling of the tube and swelling of the tube tip (Fig. 3F). Even those which entered the stigma grew very slowly. The number of pollen tubes observed in the style were considerably fewer than those of the controls. Only 20–50 pollen tubes had just reached the cut end of the 10-mm-long style by 24 h (Fig. 3G–H).

Pollen samples subjected to different treatments were used to carry out pollinations of flowers maintained on plants in order to study their ability to set fruits and seeds. There was 100% fruit set in all the pollen samples except the one treated at 45° C, in which 70% fruit set was recorded. There were also differences in the details of fruit development. The ovary on the day of anthesis measured 7–8 × 3–3.5 mm in size. There was a marked enlargement of the ovary (20.5 × 12.2 mm) within a week in flowers pollinated with fresh pollen as well as pollen subjected to high RH at 38° C. The fruits reached maximum size (22.5 × 13.5 mm) by 2 weeks and started turning brown in 4 weeks.

In flowers pollinated with pollen subjected to high

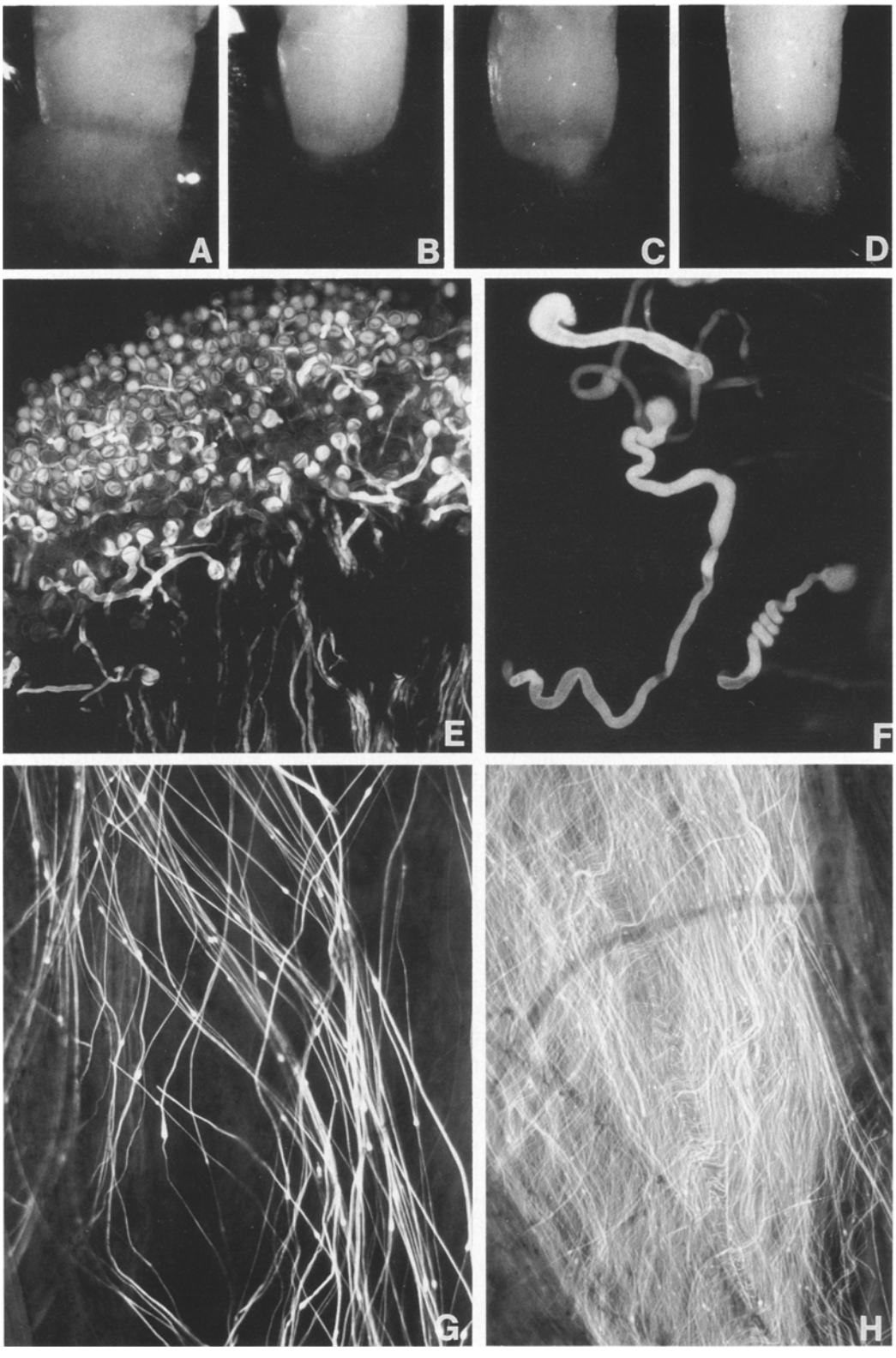

Fig. 3A–H. Germination and tube growth of pollen grains subjected to high RH and temperature stress. **A–D** Cut ends of 2-mm-long semi-vivo styles pollinated with pollen treated with high RH at laboratory temperature (**A**) and at 38° C (**B–D**). **A, B** 10 h after pollination, **C** 12 h after pollination and **D** 14 h after pollination. Very few pollen tubes have emerged in **B**, about 50 in **C** and 100 in **D** (**A**, ×12; **B–D**, ×10). Aniline blue fluorescence of stigma and style 24 h after pollination. **E–G** pollen treated with high RH at 45° C. **H** Pollen treated with high RH at laboratory temperature. Although pollen germination is satisfactory (**E**), many pollen tubes show abnormalities (**F**). The number of pollen tubes growing in the style pollinated with 45° C-treated pollen (**G**) are much fewer than those in the control (**H**). **E, G, H,** ×60; **F** ×120) —— FCR, –·– Germination

RH at 45° C, there was hardly any enlargement of the ovary in the first week, and by 2 weeks the fruits measured 15 × 9 mm. In the mature fruits, however, there was no significant difference in the size of fruits from different treatments; neither was there any obvious difference in the number of seeds. In all treatments the seeds were uniformly and compactly arranged on the enlarged placentae.

To find out if the delay in fruit initiation in flowers pollinated with pollen subjected to high RH at 45° C was due to the delay in the pollen tubes reaching the ovary, one set of pollinations were carried out with pollen subjected to high RH at either the laboratory temperature (control) or at 45° C; the stigma and style were fixed at varying intervals, and compared for pollen-tube growth. In the control a thick bundle of pollen tubes was observed throughout the length of the style and considerable number of pollen tubes and entered the ovary in 40 h after pollination. The stigma and style showed signs of senescence by 64 h, and the stigma and upper half of the style had dried by 88 h. In pistils pollinated with pollen treated at 45° C, a thick bundle of pollen tubes was observed only in the first 1 cm of the style by 40 h; there were only about 50 tubes in the subsequent 1 cm, and the longest pollen tube had reached a length of 2.5 cm. A pollen-tube bundle was observed at the base of the style in pistils fixed 88 h after pollination; even after 88 h of pollination, the stigma and style remained fresh in this treatment.

Discussion

In the present investigation pollen viability was assessed on the basis of the FCR test, as has been routinely done by many investigators (see Heslop-Harrison et al. 1984), and pollen vigour on the basis of the time taken for in vitro germination and the growth of pollen tubes in vivo using the semi-vivo technique. The results clearly show that neither high RH stress nor heat (38/45° C) given separately affect either pollen viability or pollen vigour. Interestingly the exposure of dry pollen to heat stress even at 45° C had no significant effect on pollen responses. When actively growing pollen tubes (in the medium after emerging from semi-vivo styles) of *Nicotiana sylvestris* were subjected to elevated temperatures, there is a 50% reduction in growth at 35° C and a complete inhibition of tube growth at 45° C (Kandasamy and Kristen 1989).

Even when pollen grains were subjected to both high RH and high temperature (38°/45° C) pollen viability was not affected; pollen vigour, however, was markedly reduced. Pollen grains expose to high RH at 38° C took a much longer time to germinate than pollen grains exposed to high RH at the laboratory temperature. Apparently pollen hydration results in the activation of some physiological processes that make the pollen susceptible to heat stress. At present we do not known these causative factors. The reduction in vigour was also apparent in vivo as observed in the semi-in vivo technique. The time taken for pollen tubes to emerge through the cut end of the styles in both 2-mm and 10-mm segments was much longer in pollen subjected to high RH at 38° C than in the controls. Even the division of the generative cell was delayed in heat-stressed pollen. The experiments carried out do not show conclusively whether the delay in tube emergence was due to a delay only in germination or to a delay in both germination and tube growth. However, the increase in delay of tube emergence in 10-mm styles (8 h) when compared to 2-mm styles (4 h) suggests that the delay occurs in both germination and tube growth. If the delay was only due to germination an approximately similar delay would have been expected in both 2- and 10-mm segments.

The reduction in vigour was much more pronounced when high RH was given at 45° C, the pollen grains failed to germinate in vitro. Interestingly, these pollen grains germinated on the stigma. Some factor(s) required for germination which become limiting in stressed pollen is/are made good by the stigma, as the stigma provides the best conditions for pollen germination. Although some pollen tubes after this treatment showed abnormalities, a large number of them did enter the stigma and continued to grow in the style. The growth of the pollen tubes under this treatment was much slower than in those subjected to other treatments. About 88 h were required for the pollen tubes to reach the ovary, over 40 h more than pollen tubes of the control. Therefore, the delay in initiation of fruit set in this treatment was due to a prolonged delay in the arrival of pollen tubes and consequent fertilization. Some of the pistils (30%) failed do develop into fruits and abscissed. Apparently in these pistils pollen tubes failed to reach the ovary in time to prevent the onset of senescence and eventual abscission.

The results of the present investigation and our earlier results (Shivanna and Cresti 1989) highlight the differences between pollen viability and pollen vigour in stressed pollen. As suggested by Heslop-Harrison et al. (1984), the FCR test does reflect the ability of the pollen to germinate (in vitro and/or vivo), and in most of the situations it also reflects the ability of the pollen to achieve fertilization. Thus, the FCR test is probably the best available test to assess pollen viability. However, the FCR test does not assess pollen vigour.

A few other recent investigations have brought out differences in viability, assessed on the basis of in vitro germination and pollen vigour in stored pollen. Storage has been shown to affect the ability of pollen grains to incorporate nucleic acid precursors (Bellani et al. 1984). Under many storage conditions pollen grains show a significant decline in pollen-tube growth much earlier than they show a decline in percentage germination (Kumar et al. 1988; Jain and Shivanna 1990). In the light of recent investigations highlighting the importance of pollen vigour in pollen selection and competition (see Mulcahy 1979, 1983; Ottaviano and Mulcahy 1989), it is necessary to consider pollen vigour apart from viability, particularly in stressed and stored pollen. The semi-in vivo technique or in vivo pollinations and subsequent monitoring of pollen-tube growth in the pistil through aniline blue fluorescence may be effective-

ly used, particularly in those species which do not show in vitro germination, to assess pollen vigour.

Acknowledgements. This research was carried out in the framework of the contrat CNR-RAISA. We thank Mrs. C. Faleri for technical assistance.

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